

# Apoptosis Regulators and Responses in Human Melanocytic and Keratinocytic Cells

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Apoptosis in keratinocytes is required for epidermal turnover, stratum corneum formation, and removal of ultraviolet-damaged premalignant cells. Its role in melanocyte homeostasis and transformation, on the other hand, has not been defined, although apoptosis resistance is a commonly recognized feature of melanoma. We examined the expression of apoptosis regulators in melanocytes, keratinocytes, melanoma, and HaCat cells. Melanocytic cells expressed relatively high levels of Bcl-2, Bcl-X<sub>L</sub>, Mcl-1, C-IAP-1, C-IAP-2, XIAP, Livin, and Apaf-1. The only apoptotic regulator that was differentially expressed in melanoma cells and not melanocytes was Survivin, whereas Bax was expressed in melanocytes but not in most melanoma lines. Keratinocytic cells, on the other hand, expressed high levels of FLIP and were relatively deficient in Bcl-2 family proteins. Levels of p53 were highest in HaCat cells and some of the melanoma lines, and barely detectable in

melanocytes and keratinocytes. Next, susceptibility of these cell types to apoptosis induced by ultraviolet B, the tyrosine analog 4-tert-butylphenol, and cytotoxic drugs was examined. Melanocytes were relatively resistant to ultraviolet B, whereas keratinocytes were unresponsive to 4-tert-butylphenol. Melanocytes and keratinocytes were generally less susceptible than melanoma lines and HaCat cells to etoposide, cisplatin, and staurosporine. Induction of apoptosis in these cell types was generally associated with decreased levels of Mcl-1, XIAP, and Livin, and increased levels of p53, whereas levels of other apoptotic regulators were unaltered. These results provide insights into the potential roles of apoptosis in the function and transformation of epidermal melanocytes and keratinocytes. **Key words:** 4-tert-butylphenol/Bcl-2/FLIP/keratinocyte, melanocyte/melanoma/Survivin/ultraviolet. *J Invest Dermatol* 120:48–55, 2003

**A**poptosis, or programmed cell death, is critical for epidermal homeostasis. Keratinocyte proliferation is balanced by terminal differentiation in the granular layer that leads to nuclear fragmentation (Polakowska *et al*, 1994) and caspase activation (Weil *et al*, 1999), hallmarks of apoptosis. Apoptotic keratinocyte cell death also results in the formation of the stratum corneum (Ishida-Yamamoto *et al*, 1999). In addition to these homeostatic functions in normal skin, keratinocyte apoptosis also represents an important response to environmental injury such as ultraviolet (UV) radiation. Apoptosis of keratinocytes that have sustained UV-induced DNA damage requires p53 and represents a key protective mechanism against squamous cell carcinoma by removing premalignant cells that have acquired mutations (Ziegler *et al*, 1994).

By contrast, melanocytes represent a static component of the epidermis and the role of apoptosis in basal melanocyte function and melanocytic tumor formation has not been well characterized. Nevertheless, it is generally thought that acquisition of resistance to apoptosis is important in the transition from normal melanocyte to melanoma (Gilchrist *et al*, 1999). This notion is supported by studies demonstrating apoptosis resistance of melano-

cytic nevus cells compared with melanocytes in collagen gel (Alanko *et al*, 1999) and resistance of melanoma cell lines to drug-induced apoptosis (Li *et al*, 1998). Moreover, melanoma cells *in vivo* demonstrate low levels of spontaneous apoptosis compared with other tumor cell types (Mooney *et al*, 1995; Staunton and Gaffney, 1995) and resistance to apoptosis is associated with increased metastasis in animal models of melanoma (Glinksy *et al*, 1997). The poor prognosis for patients with advanced melanoma generally results from lack of therapeutic response to conventional chemotherapy (Koh, 1991). Most chemotherapeutic drugs function by inducing apoptosis in malignant cells (Fisher, 1994), and dysfunctional apoptosis is the most likely explanation for drug resistance in melanoma (Grossman and Altieri, 2001; Satyamoorthy *et al*, 2001).

The molecular basis for apoptosis resistance in melanoma has been examined in previous studies, demonstrating in some cases increased expression of apoptotic inhibitors or loss of pro-apoptotic factors that may result in a dysfunctional apoptotic program (reviewed in Grossman and Altieri, 2001). With respect to the “extrinsic” pathway, initiated by binding of tumor necrosis factor or related cytokines (i.e., tumor necrosis factor-related apoptosis-inducing ligand) to cell surface death receptors leading to activation of caspase-8 (Ashkenazi and Dixit, 1998), several groups have reported defective death receptor signaling or downregulation in melanoma cells (Griffith *et al*, 1998; Thomas and Hersey, 1998; Ugurel *et al*, 1999). In addition, the caspase-8 inhibitor FLIP is highly expressed in melanoma (Irmeler *et al*, 1997). There has been considerable interest in the intracellular factors regulating

Manuscript received August 6, 2002; revised September 4, 2002; accepted for publication September 25, 2002

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Abbreviations: 4-TBP, 4-tert-butylphenol; IAP, inhibitor of apoptosis.

the "intrinsic" pathway, initiated by mitochondrial cytochrome *c* release and leading to activation of caspase-9 (Green and Reed, 1998). The anti-apoptotic Bcl-2, which blocks cytochrome *c* release (Adams and Cory, 1998), was found increased in melanoma compared with melanocytes in some but not all studies (Cerroni *et al*, 1995; Collins and White, 1995; Selzer *et al*, 1998; Tang *et al*, 1998). Similarly for Bcl-2-related proteins, the anti-apoptotic Bcl-X<sub>L</sub> and Mcl-1 and the pro-apoptotic Bax have been reported elevated in some but not all melanomas examined (Selzer *et al*, 1998; Tang *et al*, 1998). Deletion or inactivation by methylation of Apaf-1, a cofactor for caspase-9 (Zou *et al*, 1997), has also been described in melanoma (Soengas *et al*, 2001). It has previously been reported (Grossman *et al*, 1999a) that Survivin, a newly recognized inhibitor of apoptosis (IAP) protein (LaCasse *et al*, 1998) that blocks caspase-9 (O'Connor *et al*, 2000), was found expressed in melanoma but not normal melanocytes. Finally, another IAP protein, Livin/ML-IAP, has also been detected in melanoma cells but not melanocytes (Vucic *et al*, 2000). With the exception of these particular IAP proteins and Bcl-2 (Morales-Ducet *et al*, 1995; Kim *et al*, 2000; van den Wijngaard *et al*, 2000), however, there is little information on the expression of apoptotic regulators in melanocytes.

In this study we examine the expression of apoptotic regulatory molecules and apoptotic responses in melanocytes, melanoma cells, and keratinocytes. We found that melanocytic and keratinocytic cells exhibit different expression patterns of apoptosis regulatory proteins and differential susceptibilities to various apoptotic stimuli.

## MATERIALS AND METHODS

**Drugs and chemicals** Cisplatin (Sigma, St Louis, MO) was dissolved in dimethyl formamide and stored at 4°C. Etoposide (Sigma) was dissolved in dimethyl sulfoxide and stored at -20°C. Staurosporine (Sigma) was dissolved in dimethyl sulfoxide and stored at 4°C. 4-*tert*-butylphenol (4-TBP; Aldrich, Milwaukee, WI) was prepared monthly in 70% ethanol and stored at -20°C.

**Antibodies** Rabbit polyclonal antibodies to the following apoptotic regulators were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): Bcl-2 (n-19, sc-492), Mcl-1 (S-19, sc-819), Bcl-X<sub>L</sub> (H-62, sc-7195), FLIP<sub>S/L</sub> (H-202, sc-8347), c-IAP-1 (h-83, sc-7943), c-IAP-2 (H-85, sc-7944), and Bax (N-20). Mouse monoclonal anti-hILP/XIAP (clone 48) was obtained from Transduction Laboratories (San Diego, CA). Mouse monoclonal anti-p53 (Ab-6) was obtained from Calbiochem (San Diego, CA). Rabbit anti-serum generated against Apaf-1 (clone 846) and Livin were kindly provided by Dr Yuri Lazebnik (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and Dr Gary Kasof (AstraZeneca, Worcester, MA), respectively. Purified rabbit polyclonal antibody recognizing Survivin has been described previously (Grossman *et al*, 1999a).

**Melanocytic cells** Human melanoma lines YUSAC2, YUSIT1, YUGEN8, and LOX (Grossman *et al*, 1999a) were maintained as described previously. Normal human melanocytes were obtained from discarded foreskins, as approved by the Institutional Review Board at the University of Utah (no. 8476). Foreskins were collected in either keratinocyte growth medium (KGM)-2 (Clonetics Corp., San Diego, CA) or melanocyte medium consisting of Ham's F10 (Gibco BRL, Grand Island, NY) containing 7.5% heat-inactivated fetal calf serum (Gibco), 0.1 mM 3-isobutyl-1-methylxanthine (Sigma), 1 mM dibutyl cyclic adenosine monophosphate (Sigma), 1 μM sodium orthovanadate (Sigma), 2.5 nM cholera toxin (Sigma), 50 ng phorbol 12-myristate 13-acetate per ml (Sigma), and 1% penicillin-streptomycin-glutamine (Gibco-BRL). After scraping away excess fat and subcutaneous tissue, foreskins were floated dermis side down in 25 mg Dispase II per ml (Roche Pharmaceuticals, Nutley, NJ) in phosphate-buffered saline (PBS) at 4°C overnight. The epidermis was lifted off, placed in trypsin-ethylenediamine tetraacetic acid solution (0.05% trypsin, 0.53 mM ethylenediamine tetraacetic acid; Gibco-BRL) at 37°C, and occasionally agitated for 10 min. An equal volume of melanocyte medium was then added, epidermal fragments were carefully removed, and the cell suspension was pelleted, resuspended in melanocyte medium, and then cultured in a humidified incubator with 5% CO<sub>2</sub> at 37°C. Over the first 48 h the cell suspension was triturated twice daily to break up cell clumps

and improve cell attachment. The medium was changed every 2–3 d. After 1 wk, 0.4 mg G418 sulfate per ml (Gibco-BRL) and an equal volume of 1 M sodium hydroxide were added to the medium for 48 h to eliminate potentially contaminating fibroblasts. Melanocytes were pigmented and reacted with antibody to the melanocyte markers S-100 and Melan-A (Dako, Carpinteria, CA) by immunohistochemistry (not shown). The dibutyl cyclic adenosine monophosphate was found dispensable for melanocyte growth, and was omitted from the medium in later experiments. Early passage (1–3) melanocytes were seeded at  $3 \times 10^5$  per 35 mm dish (Falcon 353001) 1 d prior to exposure to apoptosis-inducing agents.

**Keratinocytic cells** The human keratinocyte line HaCat was maintained as described previously (Grossman *et al*, 1999b). For the preparation of normal human keratinocytes, foreskins were collected in KGM-2 medium and an epidermal suspension was obtained by trypsinization as above. Cells from two foreskins were pooled to increase yield and plating density. After diluting 1 : 2 in soybean trypsin inhibitor (10 mg per ml in PBS, Gibco-BRL), keratinocytes were pelleted, washed once in KGM-2 medium, and transferred in KGM-2 to flasks (for lysate preparation) or 35 mm dishes for apoptosis experiments. Cells were incubated as above, and medium was changed every 2–3 d until cells were used at 30–70% confluency.

**UVB irradiation** Immediately prior to irradiation, cells were washed with Dulbecco's PBS (Gibco-BRL), 1 ml PBS was added, and dishes were uncovered. The irradiation chamber contained four UVB lamps (FS20T12-UVB, National Biological Corporation, Twinsburg, OH) that emit wavelengths between 250 and 420 nm (72.6% UVB, 27.4% UVA, 0.01% UVC), with peak emission at 313 nm, according to the manufacturer. A filter (Kodacel K6808, Eastman Kodak, Rochester, NY) was placed over the dishes to block residual UVC. Dosimetry was determined using a newly calibrated UVB-500C meter (National Biological Corp.). The irradiation rate was approximately 0.2 mJ per m<sup>2</sup> per s. After UVB exposure, the medium was replaced and the recovered dishes were returned to the incubator.

**Apoptosis detection** Cells exposed to drugs or UVB were recovered by trypsinization (including nonadherents), washed in PBS, resuspended in cold 70% ethanol, and stored on ice. Apoptosis was assessed by DNA content analysis as described previously (Grossman *et al*, 1999a). Briefly, cells were ethanol-fixed and resuspended in staining solution consisting of 25 μg propidium iodide per ml (Sigma), 0.05% Triton X-100, and 100 μg RNase per ml (Gibco-BRL) in PBS. Flow cytometry was performed on a FACScan (Becton Dickinson, San Jose, CA) using Cell Quest software (Becton Dickinson).

**Western blotting** Cell lysates were prepared and protein content was measured as described previously (Grossman *et al*, 1999a). Samples (25 μg) were electrophoresed under reducing conditions at 200 V through a 5% stacking and 15% resolving acrylamide gel, and transferred on to Immobilon-P membranes (Millipore, Bedford, MA). Membrane blots were placed in PBS containing 5% nonfat milk and 0.1% Tween-20, and incubated at 4°C overnight. Blots were then incubated 2 h at room temperature in 5% nonfat milk and 0.1% Tween-20 containing primary antibodies to Bcl-2 (0.2 μg per ml), Mcl-1 (0.2 μg per ml), Bcl-X<sub>L</sub> (0.5 μg per ml), C-IAP-1 (0.5 μg per ml), C-IAP-2 (0.2 μg per ml), XIAP (0.25 μg per ml), Survivin (0.5 μg per ml), Livin (1 : 3000 dilution), FLIP (0.5 μg per ml), Apaf-1 (1 : 3000 dilution), p53 (0.1 μg per ml), or Bax (0.3 μg per ml). After washing in PBS/Tween, blots were reacted with 1 : 10,000 dilution horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse immunoglobulin (New England Nuclear, Boston, MA) in blocking solution for 30 min at room temperature. After washing in PBS/Tween, bands were visualized by enhanced chemiluminescence (Perkin-Elmer Life Sciences Inc., Boston, MA) and autoradiography. To confirm equivalent protein loading per lane, blots were subsequently washed and reacted with a 1 : 10,000 dilution of (if previously exposed to rabbit antibody) mouse anti-β-actin (clone AC-74, Sigma) or (if previously exposed to mouse antibody) rabbit anti-β-actin (Sigma), and bands visualized as above.

## RESULTS

**Expression of apoptosis regulators** We assessed the expression of a panel of apoptotic regulators in human melanocytes, melanoma cells, keratinocytes, and the keratinocyte line HaCat by western blotting. For melanocytes and keratinocytes, lysates were independently prepared from three different donors given

the possibility of individual-specific effects. We first examined apoptotic inhibitors of the Bcl-2 family, negative regulators of the mitochondrial apoptotic pathway. As shown in **Fig 1(A)**, Bcl-2 displayed a striking melanocytic-specific pattern with expression in all three melanocyte and four melanoma lines; it was barely detected in two of three keratinocyte lines and absent in HaCat cells. The Bcl-2 homolog Mcl-1 was similarly present in melanocytes and melanoma cells, and absent from keratinocytic cells (**Fig 1A**). Bcl-X<sub>L</sub>, by contrast, was seen in all cell types with strongest expression in the melanoma lines (**Fig 1A**). We then looked at members of the IAP family of caspase inhibitors. Both c-IAP1 and c-IAP2 were detected in all cell types, with strongest expression seen in melanocytes and melanoma cells and relatively weak expression in two of three keratinocyte lines (**Fig 1A**). Expression of XIAP was greatest in the melanoma lines and absent in HaCat cells, whereas melanocytes and keratinocytes expressed low but comparable levels (**Fig 1A**). Survivin was generally seen in only the transformed cells (three melanoma lines and HaCat cells) and not in either melanocytes or keratinocytes (**Fig 1A**), although low levels could be detected in the fourth melanoma line (YUSIT1) and three of seven

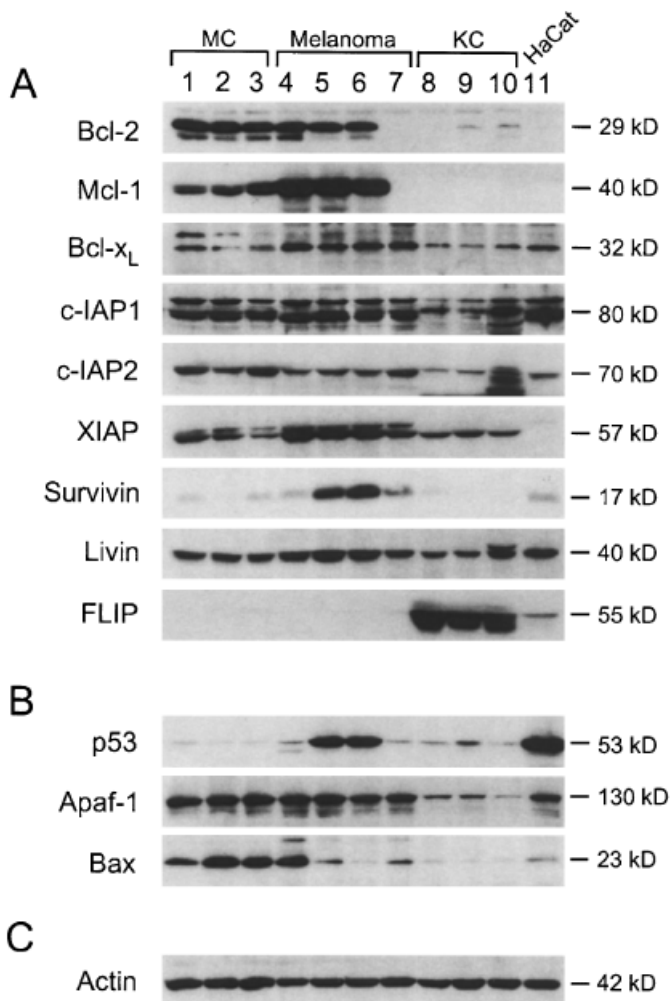
melanocyte lines on overexposed blots (not shown). Livin, on the other hand was found in all cell types, with relatively weaker expression in two of three keratinocyte lines (**Fig 1A**). The caspase-8 inhibitor FLIP was highly expressed in keratinocytes, weakly expressed in HaCat cells, and notably absent from both melanocytes and melanoma cells (**Fig 1A**).

Next, we examined expression of several pro-apoptotic regulatory molecules. The tumor suppressor p53 was highly expressed in two melanoma lines (YUSAC2, YUGEN8) and relatively weakly expressed in all other cells (**Fig 1B**). The p53-dependent caspase-9 cofactor Apaf-1 was strongly expressed in melanocytes and melanoma cells, with keratinocytes demonstrating relatively weak expression (**Fig 1B**). The Bcl-2 family member Bax was notably expressed in three melanocyte lines and YUSIT1 melanoma cells, and absent from keratinocytes (**Fig 1B**). Equivalent protein loading was confirmed for each blot by staining for actin (**Fig 1C**). Thus melanocytic cells expressed high levels of anti-apoptotic Bcl-2 and IAP family proteins, and the pro-apoptotic protein Apaf-1, whereas keratinocytic cells expressed high levels of the anti-apoptotic protein FLIP and were relatively deficient in Bcl-2 family proteins. These expression studies are summarized in **Table I**.

**Cell cycle analysis** Given recent insights into the relationship between apoptosis and cell cycle control (Evan and Vousden, 2001), we determined if there were any baseline differences in cell cycle progression or proliferation status among these cell types prior to examining the differential sensitivities of these cell types to various apoptotic stimuli. Analysis of independent subcultures of the cells used in these experiments, however, did not reveal significant differences (ANOVA,  $p > 0.05$ ) in mitotic (G<sub>2</sub>M) fractions among the melanocytic and keratinocytic cell types (**Fig 2**). There was more variability in G<sub>1</sub> fractions among the cell types, with that of HaCat being relatively lower than the rest (ANOVA,  $p = 0.001$ ), suggesting a higher percentage of HaCat cells may be in S phase.

**Apoptotic responses to UVB** UVB radiation is known to be a potent physiologic inducer of apoptosis in keratinocytes, and we confirmed that normal keratinocytes exhibited a dose-dependent (30–120 mJ per cm<sup>2</sup>) apoptotic response, as determined by nuclear propidium iodide staining and flow cytometry, which was maximal 24 h following UV exposure (**Fig 3A**). HaCat cells were similarly sensitive to UV-induced apoptosis (**Fig 3A**). Normal melanocytes, on the other hand, were markedly resistant to UV-induced apoptosis, requiring 120 mJ per cm<sup>2</sup> for a maximal response that was not evident until 48 h following exposure (**Fig 3A**). We also examined four melanoma cell lines under these same conditions. As shown in **Fig 3(B)**, peak apoptotic responses for all melanoma lines were induced at doses (60–120 mJ per cm<sup>2</sup>) comparable with that required by keratinocytes and HaCat cells, but like melanocytes, were not maximal until 48 h following UV exposure.

**Apoptotic responses to 4-TBP** Next, we examined apoptotic responses to the tyrosine analog 4-TBP, previously shown to induce apoptosis in melanocytes (Yang *et al*, 2000). Similarly, we demonstrated that 4-TBP (0.6–0.9 mM) readily induced apoptosis in melanocytes with responses peaking 48 h after initial exposure (**Fig 4A**). Keratinocytes, on the other hand, were unresponsive (**Fig 4A**). There was some variability in both melanocyte and keratinocyte responses to 4-TBP, particularly at the highest concentration tested, due to an aberrant response of cells from one of five individuals. HaCat cells were highly sensitive, with maximal responses peaking at 24 h after initial exposure (**Fig 4A**). As shown in **Fig 4(B)**, the four melanoma lines were acutely sensitive, responding to the lowest concentration (0.3 mM) tested, and apoptotic responses at each dose were generally maximal 24 h following initial exposure.

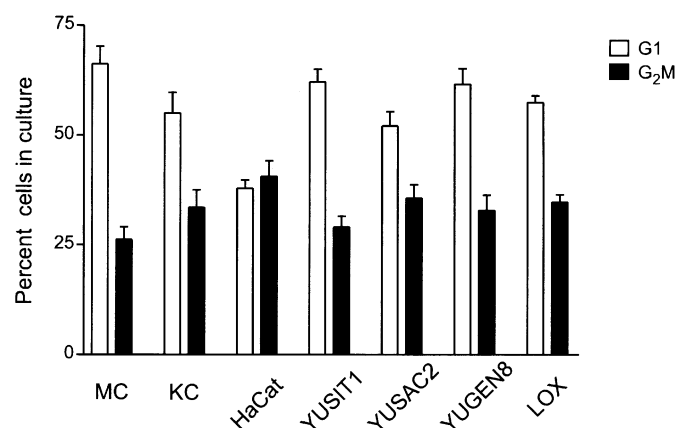


**Figure 1. Expression of apoptotic regulators.** Cell lysates (25 µg) prepared from subconfluent cultures of melanocytes (MC, lanes 1–3), melanoma lines (YUSIT1, lane 4; YUSAC2, lane 5; YUGEN8, lane 6; LOX, lane 7), keratinocytes (KC, lanes 8–10), and HaCat cells (lane 11) were subjected to western blotting using a panel of antibodies against (A) apoptosis inhibitors and (B) pro-apoptotic molecules as indicated. Approximate molecular weights of reactive species are indicated. (C) Each blot was restained with antibody to actin to confirm equivalent protein loading in each lane. A representative blot is shown.

**Table I. Summary of relative expression of apoptotic regulators<sup>a</sup>**

	Melanocytes	YUSIT1	YUSAC2	YUGEN8	LOX	Keratinocytes	HaCat
Bcl-2	++	++	++	++	0	0	0
Mcl-1	+	++	++	++	0	0	0
Bcl-X <sub>L</sub>	+	++	++	++	++	+	+
C-IAP-1	++	++	++	++	++	+	++
C-IAP-2	++	++	++	++	++	+	+
XIAP	+	++	++	++	+	+	0
Survivin	0	0	++	++	+	0	+
Livin	+	++	++	++	+	+	++
FLIP	0	0	0	0	0	++	+
Apaf-1	++	++	++	++	++	0	+
p53	0	0	+	+	0	0	++
Bax	++	++	0	0	0	0	0

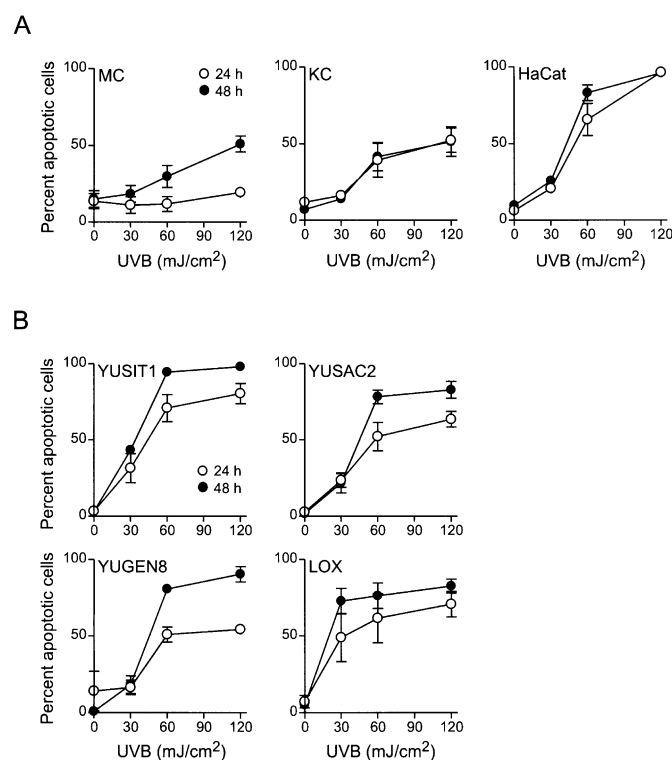
<sup>a</sup>Summary of relative expression of apoptotic regulators in untreated cultures of melanocytes, melanoma lines (YUSIT1, YUSAC2, YUGEN8, LOX), keratinocytes and HaCat cells, as determined by western blotting in **Fig 1**. Expression scored as: 0, absent or barely detectable; +, weak expression; or ++, strong expression.



**Figure 2. Cell cycle analysis.** Subconfluent untreated cultures of melanocytes (MC), keratinocytes (KC), HaCat cells, and melanoma lines (YUSIT1, YUSAC2, YUGEN8, LOX) were collected, fixed, stained with propidium iodide, and analyzed by flow cytometry. Data are expressed as percent interphase (G<sub>1</sub>, open bars) and dividing (G<sub>2</sub>M, filled bars) cells as indicated. Error bars reflect SEM from six independent subcultures.

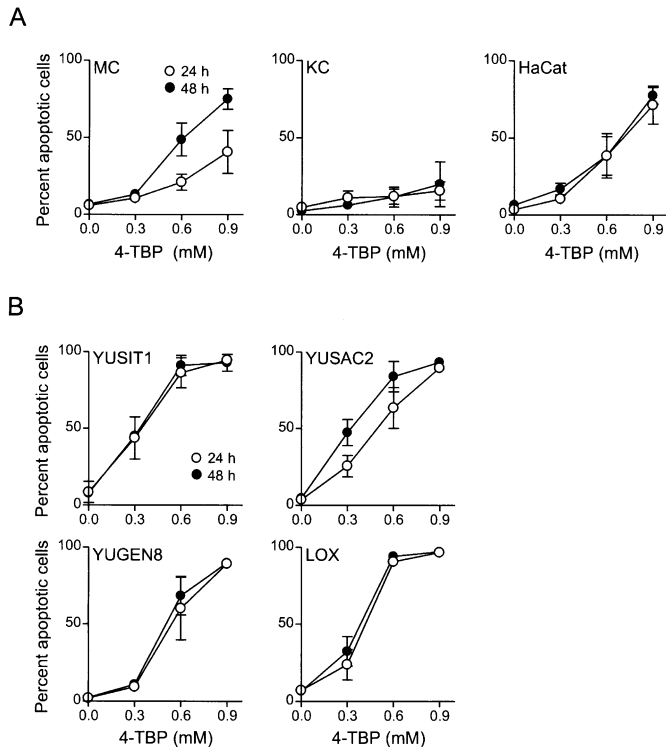
**Drug-induced apoptosis** We then determined the susceptibility of these cell types to several apoptosis-inducing drugs, each thought to initiate apoptosis by distinct mechanisms. Cells were separately incubated with increasing concentrations of drug, and apoptotic responses measured at 24 and 48 h. First, cells were exposed to etoposide (5–50  $\mu$ M), which binds topoisomerase II and blocks DNA religation. Melanocytes, keratinocytes, and two of the melanoma lines (YUSAC2 and YUGEN8) were generally unresponsive, whereas another (LOX) was minimally responsive (**Fig 5A**). By contrast, the lowest concentration (5  $\mu$ M) readily induced apoptosis in HaCat cells and the melanoma line YUSIT1 (**Fig 5A**). Second, cells were exposed to the DNA-damaging agent cisplatin (10–90  $\mu$ M). Keratinocytes and one melanoma line (YUSAC2) responded poorly, whereas other cell types exhibited moderate responses peaking 48 h after initial exposure (**Fig 5B**). Finally, apoptotic responses to staurosporine (0.2–2  $\mu$ M), an inhibitor of phospholipid/calcium-dependent protein kinase, were examined. Melanocytes, keratinocytes, and YUSAC2 melanoma cells responded minimally, whereas all other cell types exhibited robust responses generally peaking 24 h after initial exposure (**Fig 5C**). These responses are summarized, along with the other apoptotic responses observed in this study, in **Table II**.

**Apoptotic regulators during apoptotic stimulation** Finally, we investigated potential relationships in these cells between



**Figure 3. Melanocytes are resistant to UVB-induced apoptosis.** (A) Subconfluent cultures of keratinocytes (KC), HaCat cells, and melanocytes (MC) were exposed to increasing doses of UVB as indicated. Cells treated at each dose were collected and fixed either 24 (open circles) or 48 h (filled circles) after UV exposure. Data are expressed as percent apoptotic cells, derived from the sub-G<sub>1</sub> fraction obtained by flow cytometry of propidium iodide-stained cells. Error bars reflect SEM from three independent experiments, and cells (KC, MC) were derived from different donor foreskins for each experiment. (B) Subconfluent cultures of melanoma lines YUSIT1, YUSAC2, YUGEN8, and LOX were treated and analyzed as in A above. Error bars reflect SEM from three independent experiments.

apoptosis induction and expression of apoptotic regulators. For these experiments, we sought to examine each cell type under conditions in which an apoptotic response would be initiated but not maximal or complete. Based on the responses to apoptotic stimuli described above, we chose to prepare lysates from melanocytes, YUSAC2 melanoma cells, and keratinocytes 24 h after exposure to 95 mJ per cm<sup>2</sup> UVB. Lysates were simultaneously prepared from sham-treated cells to serve as controls for baseline levels of apoptotic regulators. Two



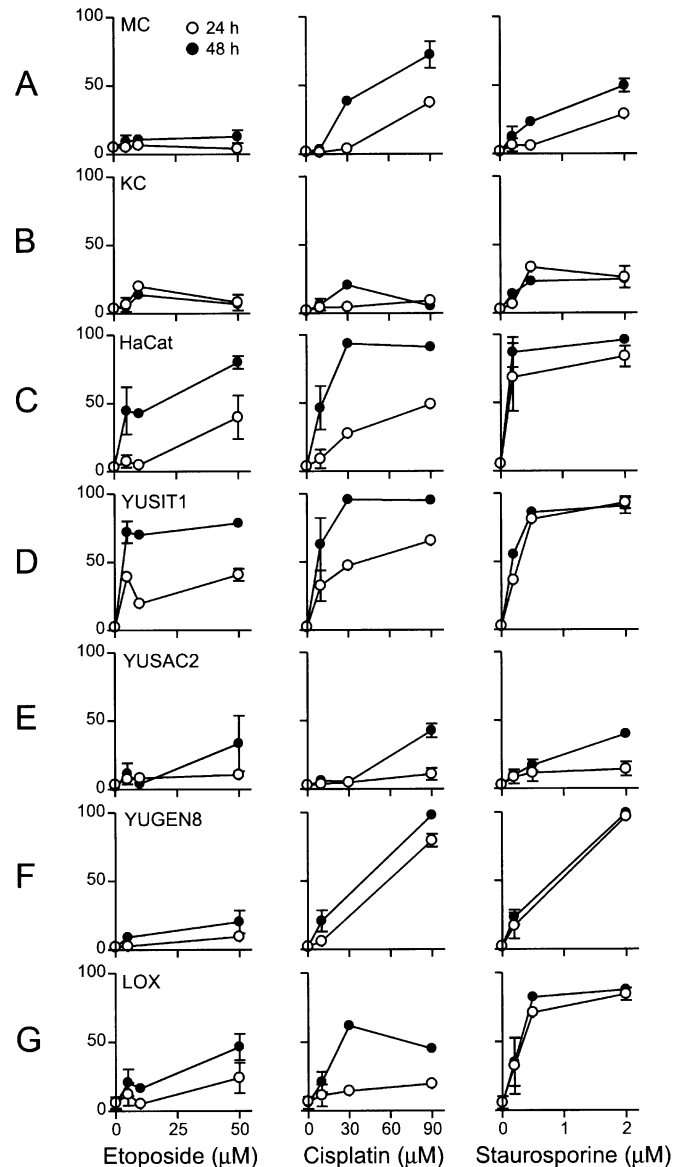
**Figure 4. Keratinocytes are unresponsive to 4-TBP-induced apoptosis.** (A) Subconfluent cultures of melanocytes (MC), keratinocytes (KC), and HaCat cells were incubated with increasing doses of 4-TBP as indicated. Cells treated at each dose were collected and fixed either 24 (open circles) or 48 h (filled circles) later. Percent apoptotic cells were determined as in **Fig 3**. Error bars reflect SEM from five independent experiments, and cells (KC, MC) were derived from different donor foreskins for each experiment. (B) Subconfluent cultures of melanoma lines YUSIT1, YUSAC2, YUGEN8, and LOX were treated and analyzed as in **A** above. Error bars reflect SEM from three independent experiments.

experiments were performed, using melanocytes and keratinocytes derived from different donors. In melanocytes, UVB-induced apoptotic stimulation was associated with reduced expression of Mcl-1, XIAP, and Livin, and induction of p53 (**Fig 6**). Keratinocytes similarly exhibited reduced expression of XIAP and Livin, and increased expression of p53. In YUSAC2 cells, UVB led to slightly decreased levels of Mcl-1, XIAP, Livin, and p53 that were associated with the appearance of putative breakdown products of 25–30 kDa (**Fig 6**). Levels of Bcl-2, Bcl-X<sub>L</sub>, c-IAP1, c-IAP2, Survivin, FLIP, Apaf-1, and Bax were not appreciably changed upon UVB exposure in any of the cell types (not shown).

## DISCUSSION

Apoptosis is known to be a key factor in keratinocyte homeostasis and squamous cell carcinoma development, yet the role of cell death in the maintenance and transformation of melanocytes is poorly understood. To define the basic apoptotic programs in these cell types, we have characterized their expression of apoptosis regulatory proteins and their responses to a variety of apoptotic stimuli. Consistent with their different functional roles, we found that melanocytic and keratinocytic cells exhibit differential expression patterns of apoptotic regulators that correlate with different susceptibilities to apoptotic signals.

Several groups (Morales-Ducret *et al*, 1995; Kim *et al*, 2000; van den Wijngaard *et al*, 2000) had previously demonstrated Bcl-2 expression in melanocytes. These observations were confirmed, and



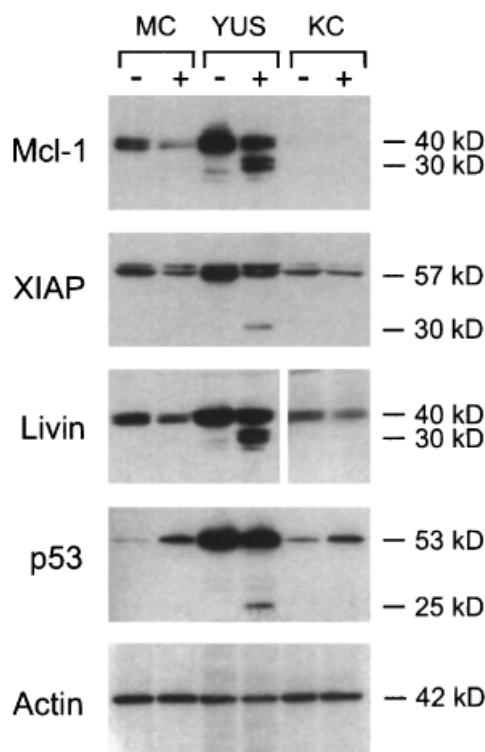
**Figure 5. Drug-induced apoptosis.** Subconfluent cultures of (A) melanocytes (MC), (B) keratinocytes (KC), (C) HaCat cells, and (D–G) melanoma lines YUSIT1, YUSAC2, YUGEN8, and LOX were exposed to increasing concentrations of etoposide, cisplatin, or staurosporine as indicated. Cells treated at each dose were collected and fixed either 24 (open circles) or 48 h (filled circles) later. Percent apoptotic cells were determined as in **Fig 3**. Error bars reflect SEM from two or three independent experiments, and cells (KC, MC) were derived from different donor foreskins for each experiment.

we also found that melanocytes expressed relatively high levels of additional Bcl-2 family proteins Bcl-X<sub>L</sub> and Mcl-1, as well as the pro-apoptotic Bax, whereas keratinocytic cells were relatively deficient in these proteins. Keratinocytic cells, in contrast to melanocytic cells, expressed high levels of the anti-apoptotic protein FLIP. We did not detect significant FLIP expression in either melanocytes or melanoma cells, although Irmeler *et al* (1997) reported FLIP expression in both melanoma tumors and cell lines. In the three of four melanoma lines examined, levels of Bcl-2 were comparable with that in melanocytes, whereas both Mcl-1 and Bcl-X<sub>L</sub> were generally elevated. Bax was highly expressed in only one of four melanoma lines. These findings are consistent with previous expression studies in melanoma lesions on Bcl-2 (Selzer *et al*, 1998; Tang *et al*, 1998), Bcl-X<sub>L</sub> (Selzer *et al*, 1998; Leiter *et al*,

**Table II. Summary of cell sensitivity to apoptotic stimuli<sup>a</sup>**

	Keratinocytes	HaCat	Melanocytes	YUSIT1	YUSAC2	YUGEN8	LOX
UVB	++	+++	+	++	++	++	++
4-TBP	0	+	++	+++	+++	+++	+++
Etoposide	0	++	0	++	0	0	+
Cisplatin	0	++	++	++	+	++	++
Staurosporine	+	+++	+	+++	+	+++	+++

<sup>a</sup>Summary of relative responses of melanocytes, keratinocytes, HaCat cells, and melanoma lines (YUSIT1, YUSAC2, YUGEN8, LOX) to various apoptotic stimuli examined in this study. Responses to UVB and 4-TBP are derived from **Figs 3 and 4**, respectively. Responses to etoposide, cisplatin, and staurosporine are derived from **Fig 5**. Apoptotic response scored as: 0, no response at 24 h; +, weak response peaking at 48 h; ++, moderate response peaking at 48 h; or +++, strong response peaking at 24 h.



**Figure 6. Expression of apoptotic regulators during apoptotic induction.** Cell lysates (25 µg), prepared from subconfluent cultures of melanocytes (MC), YUSAC2 melanoma cells (YUS), and keratinocytes (KC) 24 h following sham treatment (–) or exposure to 95 mJ per cm<sup>2</sup> UVB (+), were subjected to western blotting for apoptosis inhibitors and proapoptotic molecules as indicated. Adequate visualization of the Livin blot for keratinocytes required relatively longer exposure time than that for melanocytes and YUSAC2, thus optimal exposures are shown for each. Approximate molecular weights of reactive species are noted. Blots were restained with antibody to actin to confirm equivalent protein loading in each lane. A representative blot is shown. Results are representative of two independent experiments performed.

2000), Mcl-1 (Tang *et al*, 1998), and Bax (Selzer *et al*, 1998; Leiter *et al*, 2000). Raisova *et al* (2001) have shown that susceptibility to death receptor signaling in melanoma lines correlates with a high Bax/Bcl-2 ratio. We did not observe such a correlation among the lines studied here with sensitivity to various apoptotic stimuli, but it is intriguing that the LOX melanoma line was responsive to lower doses of UVB and cytotoxic drugs and was the only line that did not express Bcl-2 or Mcl-1. Of all the apoptotic regulators examined, Bax was the only protein consistently expressed in melanocytes and not melanoma cells.

Melanocytic cells also consistently expressed multiple IAP proteins, including C-IAP-1, C-IAP-2, XIAP, and Livin. The c-IAP proteins were expressed at roughly equivalent levels in melanocytes and melanoma cells, whereas XIAP and Livin were

found at higher levels in melanoma cells compared with melanocytes. Initial studies (Vucic *et al*, 2000; Kasof and Gomes, 2001) on Livin (or ML-IAP) noted expression in melanoma cells but not melanocytes (Vucic *et al*, 2000). Our ability to detect Livin expression in melanocytes may be due to our use of a polyclonal reagent that would be expected to react with both isoforms that have been reported (Ashhab *et al*, 2001). Consistent with previous work (Grossman *et al*, 1999a), the only apoptotic regulator that was generally expressed in melanoma cells and not melanocytes was the IAP protein Survivin, suggesting that it may be important at a relatively later stage in melanocyte transformation.

The pro-apoptotic protein p53 was highly expressed in HaCat cells and two of the melanoma lines, but not in either melanocytes or keratinocytes. High levels of p53 generally reflect mutant protein, and it is known that both alleles of the p53 gene are mutated in HaCat cells (Lehman *et al*, 1993). It is likely that p53 mutation accounts for the high levels detected in YUSAC2 and YUGEN8 melanoma lines. Finally, although Soengas *et al* (2001) reported loss of Apaf-1 in melanoma lesions and 10 of 19 melanoma lines, we found Apaf-1 consistently expressed in both melanocytes and all four melanoma cell lines.

UVB radiation is the primary mutagen involved in nonmelanoma skin cancer (Grossman and Leffell, 1997) and a physiologic apoptotic stimulus. Most premalignant keratinocytes with UVB-induced mutations are eliminated rapidly by apoptosis, or lost slowly through normal epidermal turnover. Consistent with this scenario and previous observations (Jost *et al*, 2001), UVB produced a rapid apoptotic response in both normal keratinocytes and the keratinocyte line HaCat. By comparison, we found that normal melanocytes were relatively resistant to UVB-induced apoptosis. Notable distinctions between keratinocytes and melanocytes are that the former are present in large numbers and chronically renewed in the epidermis, whereas the latter sparsely populate the epidermis and are thought to be long-lived (Gilchrist *et al*, 1999). Melanocytes could be viewed as less dispensable than keratinocytes, and thus it makes teleologic sense that they would have a much higher threshold than keratinocytes for UVB-induced apoptosis.

The blunted apoptotic response of melanocytes to UVB was not indicative of resistance to all apoptotic stimuli, as these cells were quite sensitive to 4-TBP-induced apoptosis. We found that normal keratinocytes, on the other hand, were refractory to 4-TBP, consistent with a previous report (Yang *et al*, 2000). Yang *et al* (2000) also first reported that melanocyte cytotoxicity induced by 4-TBP represented apoptotic cell death, demonstrating DNA fragmentation and phosphatidylserine relocation in melanocytes exposed to 4-TBP. Depigmenting agents, such as 4-TBP, were originally proposed to act as substrates for tyrosinase (McGuire and Hendee, 1971). The mechanism by which 4-TBP induces apoptosis in melanocytes, however, is unclear and apparently not mediated by tyrosinase (Yang *et al*, 2000). The A2b adenosine receptor may be involved, as Le Poole *et al* (1999) showed it is upregulated in 4-TBP-treated melanocytes and not keratinocytes.

A curious feature of both UVB and 4-TBP apoptotic responses is that melanoma and HaCat cells were more sensitive, respectively, than normal melanocytes and keratinocytes. This was

somewhat unexpected, as we would have predicted that the malignant cells would be more resistant to these apoptotic stimuli than the untransformed cells from which they were derived. One possibility is that long-term culture of cell lines selects for clones with a proliferative advantage *in vitro* that may be attained at the expense of apoptotic resistance. The mechanistic basis for increased susceptibility to UVB/4-TBP-induced apoptosis in melanoma and HaCat cells is unclear, however, as we did not consistently find increased expression of pro-apoptotic molecules or decreased expression of apoptotic inhibitors compared with normal melanocytes and keratinocytes. Similarly, HaCat cells were consistently more sensitive to drug-induced apoptosis than normal keratinocytes. When comparing drug responses of the melanoma cell lines with normal melanocytes, on the other hand, no consistent pattern emerged. For example, two of four melanoma lines responded to etoposide, whereas melanocytes were unresponsive; melanocytes responded better to cisplatin than two of the four melanoma lines tested; and finally, three of the four melanoma lines responded better to staurosporine than did melanocytes. The susceptibility of these cell types to drug-induced apoptosis was not related to the number of proliferating cells in culture, as they displayed comparable G<sub>2</sub>M fractions despite very disparate responses.

The expression and activity of apoptotic regulatory proteins maintains a critical balance in cells, with anti-apoptotic and pro-apoptotic regulators potentially exerting influence at multiple control points in apoptotic pathways. Although melanocytes and keratinocytes displayed almost reciprocal expression patterns for many of these proteins, we found that apoptotic stimulation had similar effects in both. Specifically, UVB exposure in both cell types was associated with downregulation of two apoptotic inhibitors (XIAP and Livin) and upregulation of a pro-apoptotic molecule (p53). Thus these changes are consistent with the known functional activities of these particular regulatory proteins. It is intriguing that only selected members of the IAP family, namely XIAP and Livin, were altered, whereas others such as the C-IAPs and Survivin were not. Similarly, it is unclear why apoptotic stimulation of melanocytes, for example, would result in selective reduction in Mcl-1 without affecting other Bcl-2 family proteins. It is interesting that in YUSAC2 cells, UVB exposure was associated with what appears to be limited proteolysis or cleavage of these regulators, as was recently described for XIAP in tumor necrosis factor-related apoptosis-inducing ligand-stimulated melanoma cells (Zhang *et al*, 2001). It is also possible that the lower band in these blots may represent induction of smaller cross-reactive proteins.

In conclusion, the differences reported here between melanocytes and keratinocytes, with respect to expression of apoptotic regulators and apoptotic responses, suggest that these cell types are endowed with different apoptotic programs to meet different functional needs in the skin. The relative deficiency of apoptotic inhibitors in keratinocytes may maintain a low apoptotic threshold that is required to sustain rapid turnover and efficiently remove damaged cells. The broad expression of apoptotic inhibitors in melanocytes, on the other hand, may serve to maintain their longevity, but this could come at the expense of retaining damaged cells with the potential of developing into melanoma. Our data do not support the notion of reduced susceptibility to apoptosis in the progression from melanocyte to melanoma, but rather suggest that the apoptosis resistance characteristic of melanoma clinically may merely reflect an anti-apoptotic state that is well established in precursor melanocytes. The ability to precipitate apoptosis, however, in malignant melanocytes and keratinocytes by expressing p53 (Yamashita *et al*, 2001) or Apaf-1 (Soengas *et al*, 2001), or by blocking individual apoptosis inhibitors of either the Bcl-2 (Olie *et al*, 2002) or IAP (Grossman *et al*, 1999a; Grossman *et al*, 1999b; Kasof and Gomes, 2001) families, suggests that the apoptotic balance in these cells is easily perturbed and thus could potentially be exploited for therapeutic gain.

We thank Drs Yuri Lazebnik for Apaf-1 antibody and Gary Kasof for Livin antibody. We also thank Dr Sancy Leachman for critically reading the manuscript. Supported by NIH grant KO8AR48618, the Huntsman Cancer Foundation, and a Fellowship-to-Faculty Transition Award from the University of Utah funded in part by the Howard Hughes Medical Institute.

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